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Isolation and screening of marine associated bacteria from Tamil Nadu, Southeast coast of India for potential antibacterial activity

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Abstract - The bacteria associated with living surfaces are rich sources of bioactive metabolites. In the present study, 182 heterotrophic epibacterial colonies, isolated from seaweeds (44%), ascidians (30.2%), barnacles (10.4%) and molluscan egg mass (15.4%), were subjected to high throughput screening by cross streaking method against six human pathogenic bacteria, *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio cholerae, Proteus mirabilis* and *Klebsiella pneumoniae*. The 137 epibacterial isolates, which showed activity against at least one human bacterial pathogen in cross streaking method, were further cultured and the ethyl acetate extract of the culture broth (100 μg/disc) was assayed for antibacterial activity through disc diffusion method. The four epibacterial colonies, BR1, EM13, EM14 and PC4, isolated from the barnacle *Balanus amphitrite*, seaweed *Enteromorpha compressa*, and ascidian *Polyclinum constellatum* showed broad spectral antibacterial activity.

Key words: high throughput screening, antibacterial activity, marine bacteria, cross streaking method, ascidian, barnacle.

INTRODUCTION

In the ocean, the bacterial populations typically range from 10^3 to 10^6 per millilitre with as many as 10^9 per millilitre in marine sediments (Austin, 1988). Bacteria help regulate rates of organic matter mineralisation, nutrient cycling and energy transfer in aquatic environments (Azam and Worden, 2004) and they produce a variety of metabolites, some of which can be used for drug development (Fenical, 1993; Grossart *et al.,* 2004). Bacteria living in complex associations with animals are often proposed to be the real producers of 'invertebrate' metabolites (Proksch *et al.*, 2002). But, the distributions of marine bacteria are poorly known. The important microhabitats for marine bacteria are the animate and inanimate surfaces and internal spaces of invertebrate animals. Marine invertebrates are laden with bacterial symbionts, often in high density (40% of weight) or diversity (hundreds of colonies/animal) (Schmidt, 2005).

Marine epibiotic bacteria growing on the surface of seaweeds and other invertebrates live in a highly competitive environment where space and access to nutrients are limited. Bioactive compound production in these bacteria could be attributed to the competition among them for space nutrition (Burgess *et al.*, 1999). The bacteria present on the marine surfaces can produce secondary metabolites which inhibit the settlement of other bacteria (Holmstrom and Kjelleberg, 1994). A study of bacteria isolated from marine algal surfaces indicated that the incidence of antibiotic producing colonies from this habitat was 20 per cent whereas that from seawater was only a few per cent (Lemos *et al.*, 1985).

Marine plants and animals are well known to have developed symbiotic relationships with numerous microorganisms. Symbiosis between bacteria and primitive organisms were an absolute necessity for the diversity and evolution of multicelled eukaryotic organisms (Margulis, 1993). Antibacterial activity among marine bacteria is a well-known phenomenon and has been demonstrated in a number of studies (Isnansetyo *et al.,* 2003; Uzair *et al.,* 2006). However, their ecological role and degrees of adaptation to the marine environment is largely unknown (Bush, 2004).

In the present study, bacteria isolated from various living marine surfaces such as, seaweeds, ascidians, molluscan egg masses and barnacles were subjected to high throughput screening against human bacterial pathogens.

MATERIALS AND METHODS

Seaweeds and barnacles, collected from Tuticorin (lat. 8°45′ N and long. 78°13′ E), Mahabalipuram (lat. 12°37′ N and long. 80°14′ E) and Kovalam (lat. 12°47′23.41″ N and long.

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80°14′52.72″ E) coasts and that of ascidians and molluscan egg masses from Tuticorin coast of Southeast coast of India were transported to the laboratory for the isolation of surface associated bacteria. The associated bacterial isolates were selected based on colony morphology.

Isolation of seaweed associated bacteria. The seaweeds, *Chaetomorpha linoids*, *Ulva lactuca*, *Enteromorpha compressa* and *Gracilaria edulis*, transported to the laboratory in sterile plastic bags, were first rinsed with sterile seawater for a few seconds to remove the loosely attached bacteria (Lemos *et al.*, 1985). One gram of seaweed was weighed and homogenised with 9 ml of sterile seawater, vortexed, serially diluted and 0.1 ml aliquots were plated on Zobell Marine agar (HiMedia, Mumbai, India) and Actinomycetes agar (HiMedia) in triplicate. This procedure was repeated in duplicate for each species (Wahl *et al.*, 1994, Chelossi *et al.*, 2004). The plates were incubated at room temperature for 7 days. The colonies were counted and expressed as Colony Forming Units (CFU) per g. The individual bacterial strains were isolated by repeated streaking and stored in Zobell Marine agar slants at 4 °C until further study.

Isolation of ascidian associated bacteria. The associated bacteria from four species of ascidians, *Polyclinum constellatum, Phallusia nigra, Eudistoma viridae* and *Didemnum psammathodes*, collected separately in sterile polythene bags, transported to the laboratory and washed gently with sterile seawater, was isolated by homogenising one gram of tissue in 9 ml sterile seawater, vortexed, serially diluted and 0.1 ml aliquots were inoculated onto B_1 medium agar plates (2.5 g peptone, 1.5 g yeast extract, 1.5 ml glycerol, 17 g agar, 750 ml filtered seawater, 250 ml deionised water) (Wahl, 1995). The plates were incubated at room temperature for 7 days and the colonies were counted and expressed as CFU per g. The individual bacterial colonies were isolated by repeated streaking and stored in Zobell Marine agar slants at 4 °C until further study.

Isolation of barnacle associated bacteria. The barnacle associated bacteria was isolated from *Balanus amphitrite*. The whole animal with hard cement covering was crushed and one gram was homogenised by mortar and pestle with 9 ml sterile seawater, vortexed, serially diluted and 0.1 ml aliquots were plated on Zobell Marine agar and Actinomycetes agar. Triplicates were maintained and the plates were incubated at room temperature for 7 days. The individual bacterial strains were isolated by repeated streaking and stored in Zobell Marine agar slants at 4 °C until further study.

Isolation of molluscan egg mass associated bacteria. The egg mass of *Cyprae* sp., collected carefully without damaging the eggs and brought to lab, was homogenised with 9 ml of sterilised seawater, serially diluted and the bacterial colonies were isolated by plating 0.1 ml aliquots onto Zobell Marine agar and Actinomycetes agar. Triplicates were maintained and the plates were incubated at room temperature for 7 days. The individual bacterial colony was isolated by repeated streaking and stored in Zobell Marine agar slants at 4 °C until further study.

High throughput screening. The preliminary screening of bacteria were carried out by following the method of Strahl *et al.* (2002). The marine isolates were streaked on to TSA plates (Tryptons Soya agar + 1% NaCl) and incubated at room temperature for 5 days. Test colonies of human pathogens such as *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Vibrio cholerae, Proteus mirabilis* and *Klebsiella pneumoniae* were then streaked perpendicular to the marine colonies and incubated overnight. The activity was indicated by the inhibited growth of pathogenic colony as compared to a control plate (Lemos *et al.,* 1985).

In the secondary screening, the metabolites of active isolates were assayed against the same human pathogens by standard disc diffusion method. The active marine colonies were inoculated onto 100 ml of Zobell Marine broth separately. The inoculated colonies were broth cultured in a shaker at 290 rpm for 5-7 days at room temperature, centrifuged, filtered and then the supernatant was extracted employing liquid-liquid extraction (Gailliot, 1998). Equal volume of ethyl acetate was added to the broth and stirred for 30 min using a magnetic stirrer. The two phases were then separated in a separating funnel and the solvent phase was concentrated by evaporation. The concentrated crude extracts and ethyl acetate solvent control were then impregnated on to sterile Whatman 6 mm discs (approximately 100 µg/disc) and antibacterial activity was assayed following the disc diffusion assay (Acar, 1980, Becerro *et al.*, 1994, Murugan and Santhana Ramasamy, 2003). The inhibition zone was measured from border of the disc to the edge of the clear zone in mm.

RESULTS AND DISCUSSION

The first attempt to locate antibacterial activity in marine organisms was initiated around 1950s (Burkholder and Burkholder, 1958). Though the occurrence of unusual natural products from marine invertebrates and their associated bacteria has been widely reported (Burgess *et al*., 1999), their relative biological function has not been studied much (Pawlik, 1992). In the marine environment, most of the microorganisms form associations with marine eukaryotes (Polz *et al.* 1999, Fieseler *et al.*, 2004). These kinds of associations may provide nutrients or dietary cofactors to the host or perhaps molecules that protect the host from 'infection' by other eukaryotes (Holmstrom and Kjelleberg, 1994, Spragg *et al.*, 1998).

In the present study, a total of 182 morphologically distinct heterotrophic associated bacterial colonies were isolated. Of these, most of the epibiotic bacteria were non-pigmented. The total viable bacterial count varied from 3 x 10^6 to a lowest of 3 x 10^3 CFU/cm^2/g. A total of 80 colonies were isolated from seaweeds represented by 32 (40%) from *Ulva lactuca*, 11 (14%) from *Chaetomorpha linoids*, 22 (27%) from *Gracilaria edulis* and 15 (19%) from *Enteromorpha compressa*.

In ascidians, the total associated bacterial count ranged from 100 x 10^4 to 23 x 10^3 CFU/g and the highest number of 16 (29%) colonies were isolated from the simple ascidian *Phallusia nigra* (black ascidian) followed by colonial ascidians such as *Polyclinum constellatum*, *Eudistoma viridae* and *Didemnum psammathodes* with 13 (24%), 14 (25%), 12 (22%) colonies respectively. In barnacle *Balanus amphitrite*, associated bacterial density of 11 x 10^4 CFU/g was observed and 19 colonies were isolated. Bacterial density of 9 x 10^4 was observed in the egg masses of *Cyprae errones* and a total of 16 and 12 from *Sepia pharaonis* bacterial colonies were isolated. The percentage of bacteria showing antibacterial activity and broad spectral activity from different living marine sources is shown in Fig. 1.

Out of the total of 182 associated bacterial isolates, 113 showed activity against at least one bacterial pathogens in the cross streaking method (data not shown). The extracts of culture supernatants of the active isolates, represented by 55 (49%) from seaweeds, 34 (30%) from ascidians, 9 (8%) from barnacle

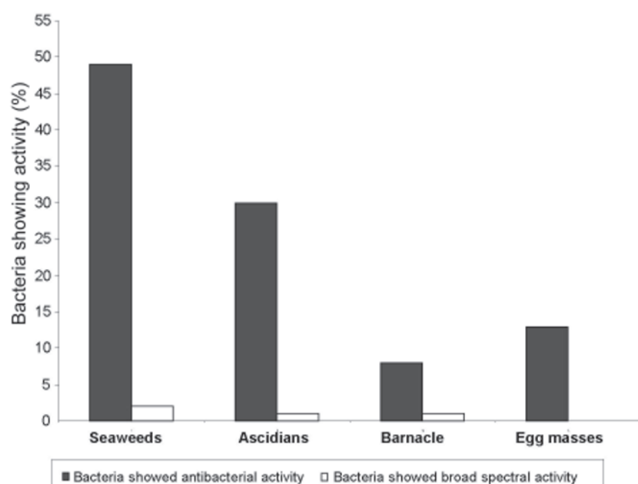

FIG. 1 - Percentage of bacteria showing activity and broad spectral activity of marine surfaces.

and 15 (13%) from molluscan egg mass, were subjected to secondary screening through disc diffusion assay and the results of isolates showing activity at least against two bacteria or higher inhibition against one bacteria are presented in Table 1.

In disc diffusion method, the bacteria isolated from the seaweed *Enteromorpha compressa* EM13 and EM14 exhibited broad spectral activity against all the six pathogens. The colony EM13 showed higher activity than EM14 with 20 mm inhibition zone against *Pseudomonas aeruginosa* and *Escherichia coli.* The bacterial colony UL22 isolated from *Ulva lactuca* showed activity against four pathogens. This was in line with the observation of Lemos *et al.* (1985) who have recorded antibiotic activity of the epiphytic marine bacteria from intertidal seaweeds with a zone of inhibition of greater than 10 mm against *Staphylococcus aureus*, *Escherichia coli*, *Alcaligens faecalis,* etc.

In the present study, higher percentage of bacteria was isolated from seaweeds especially from *Ulva lactuca*. This observation coincided with Lemos *et al.* (1985) who have isolated 224 epiphytic bacterial colonies from intertidal seaweeds and out of which, 38 colonies (16.9%) displayed antibacterial activity. Spragg *et al.* (1997) isolated 51 colonies from marine algae *Fucus resiculosus* and in that 13 (25%) showed activity against Methicillin Resistant *Staphylococcus aureus* (MRSA). Jayanth *et al.* (2002) also reported similar observation in their study on antagonistic marine bacteria against pathogenic bacteria. It is to be noted that the field observation showed that this *Ulva lactuca* was free from macrofouling. However, the presence of the associated (epi) bacteria may be attributed to their role in preventing or helping the host organisms from biofouling (Holmstorm *et al*., 1992, 1998). The antibacterial assay in the present study indicated the higher inhibitory activity of bacteria isolated from *Enteromorpha compressa* (EM13, EM14).

From the ascidians, 55 colonies were isolated. Of these 61.8% were active in cross streaking method and 25.5% in disc diffusion assay. The colony PC4, isolated from the ascidian *Polyclinum constellatum*, showed activity against five pathogens. Ascidians have already been reported to be the rich source of nitrogen compounds with a wide range of biological activities (Biard *et al.*, 1994). The ethyl acetate extract of bacteria PC4 from *Polyclinum constellatum* exhibited broad spectral activity except against *Proteus mirabilis*. Compared to seaweeds, the associated bacterial density was low in ascidians. The activity observed with ascidians associated bacteria could well be substantiated by the report (James *et al.,*1996) of bactericidal activity of a novel protein produced by a biofilm-forming marine bacterium, D2 (*Pseudoalteromonas tunicata*) isolated from the surface of the tunicate *Ciona intestinalis*, against a wide variety of marine and medical bacterial isolates

The molluscan egg mass associated bacteria did not show any promising activity against all the six pathogens. The low density of associated bacterial colonies in the egg mass of marine molluscs could be attributed to the known phenomenon that a number of marine molluscs protect their eggs with natural products (Pawlik, 1988). The present study was supported by Benkendorff (1999) who have reported antimicrobial activity in the egg masses of 34 species of marine molluscs including six species of muricidae. The observation of Santhana Ramasamy and Murugan (2005, 2007) of antibacterial activity in molluscan egg masses against biofilm bacteria and human pathogenic bacteria also corroborated the present observation.

The highest zone of inhibition of 21 mm with broad spectral activity against all human pathogens was observed against *Escherichia coli* in BR1 (*Balanus amphitrite*) extract. Modulation of metamorphosis in barnacles in response to cues of biological origin has been established and associated bacteria may also have a role in such modulations (Lidita *et al*., 2003). Three neutral sugars, D-mannose, D-glucose and D-galactose, form the most common constituents of bacterial exopolysaccharides from both marine and freshwater environments (Sutherland, 1980). The compositions of bacterial exopolymers, which act as chemical cues, have been shown influence subsequent settlement by invertebrate larvae (Maki *et al*., 1988, 2000). So, the bacteria associated with barnacle may play a similar role and the antibacterial activity could be explained to the production of exopolymers.

The activity in ethyl acetate extracts were, in general, higher than Zheng *et al*. (2005) who have reported moderate activity (1-5 mm) in the ethyl acetate extract of two sponge associated bacteria against pathogenic bacteria. The activity in culture supernatants of the associated colonies indicated the production of exocellular metabolites. The activity was in line with the antagonistic activity observed in cross streaking assay. So, there exist possibilities that the metabolites produced by the associated colonies may be toxic in nature. However, it has been predicted that the metabolite produced by the associated colonies may have an ecological role to play with and hence, may be inhibitory and not toxic. This was substantiated by the observation by Costerton (1974) that extracellular polymers produced by most of the microbes, augment their ability to compete and survive in changing environmental conditions by altering the physical and biogeochemical micro-environment around the cell. The role of microbial exopolymers in the ocean has been reviewed extensively (Wolfaardt *et al*., 1999).

In this study, BRI colony from *Balanus amphitrite,* EM13, EM14 from *Enteromorpha compressa* showed promising antibacterial activity against all six pathogens. The colony PC4 also exhibited promising activity against all pathogens, except *Proteus mirabilis*. The activity observed against human bacterial pathogens was substantiated by the observation of antibacterial activity of five epiphytic colonies against human pathogenic bacteria *Enterobacter faecalis*, *Staphylococcus aureus* etc. by Chelossi *et al*. (2004). Further, isolation, purification and structural elucidation of these associated bacterial extracts would through more light on the nature and potential of these antibacterial compounds against human pathogens.

TABLE 1 - Antibacterial activity of crude ethyl acetate culture extracts of associated bacteria on human bacterial pathogens.

Associated bacterial isolates	Zone of inhibition in (mm)					
	Staphylococcus aureus	*Klebsiella pneumoniae*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Vibrio cholerae*	*Proteus mirabilis*
Seaweeds associated						
UL 3	7	-*	-	2	-	-
UL 14	-	T**	-	-	T	T
UL 19	2	T	1	-	-	-
UL 22	3	3	3	-	4	-
UL 31	T	T	T	-	T	-
CL 4	2	2	-	T	-	-
CL 6	3	-	-	2	-	1
GE 1	3	-	3	-	5	-
GE 2	-	2	-	3	-	-
GE 3	-	7	-	-	-	1
EM 1	-	3	-	-	5	-
EM 2	9	-	2	2	-	-
EM 5	2	2	-	T	-	-
EM 8	-	7	-	-	4	-
EM 11	4	3	6	-	5	-
EM 12	-	-	2	-	3	-
EM 13	8	10	20	20	17	12
EM 14	2	2	3	5	5	4
EM 15	2	-	-	4	-	-
Ascidians associated						
PC 4	20	10	18	17	19	-
EV 7	-	T	2	-	2	-
DP 1	1	1	-	-	-	T
DP 6	-	-	2	2	-	-
Barnacle associated (*Balanus amphitrite*)						
BR 1	17	19	21	18	10	15
BR 2	-	8	-	1	T	-
BR 3	-	-	-	4	-	-
BR 10	-	T	2	-	T	-
BR 13	-	4	-	-	-	3
Molluscan egg mass associated						
CE 1	-	1	-	6	-	-
CE 4	2	-	2	-	-	-
SP 2	-	-	-	2	2	-
SP 3	-	-	3	-	2	-
SP 12	2	2	-	T	-	-
Control	-	-	-	-	-	-

UL: *Ulva lactuca*, CL: *Chetomorpha linoids*, GE: *Gracilaria edulis*, EM: *Enteromorpha compressa,* PC: *Polyclinum constellatum*, EV: *Eudistoma viridae*, DP: *Didemnum psammathodes*, CE: *Cyprae errones*, SP: *Sepia pharaonis*.
* -: no activity, ** T: trace.

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